Peptides from the hPTH(1-37) Sequence

The present invention relates to peptides from the sequence of hPTH(1-37), and the use of said peptides in the preparation of an agent for diagnosing biologically active hPTH.

Human parathyroid hormone (hPTH), a linear polypeptide having 84 amino acids, plays an important role in the regulation of the calcium metabolism. The metabolism of this hormone gives rise to a large number of C-terminal fragments, the biological functions of which have not yet been elucidated. The hPTH(1-37) has been established as a circulating N-terminal fragment (EP-A 0 349 545). This fragment has the full biological activity of the entire hormone. However, upon loss of the first amino acid, serine, the activity significantly decreases and is lost completely without the first two amino acids, serine and valine.

Serum levels in the range of 10^{-12} mol/l are measured for the intact hormone hPTH(1-84) and for the N-terminal fragment. Immunological measuring procedures are employed to determine such low concentrations. Here, the most valid results are provided by measuring procedures according to the double antibody or sandwich principle (e.g., the two-site radioimmunometric assay, IRMA, or the sandwich enzyme-linked immuno sorbent assay, Sandwich ELISA). For hPTH(1-84), such assays are commercially available. For the measurement of hPTH(1-34), an assay according to the double antibody principle is not known.

Here, two antibodies are required. In order to avoid mutual steric hindrance, they must be capable of recognizing antigen epitopes located at a sufficient distance from each other. When immunizing using the intact antigen, a heteroge-

neous mixture of various antibodies is obtained, which first must be subjected to an expensive purification in order to conduct a sandwich assay. According to theoretical calculations by B.A. Jameson and H. Wolf, The Antigenic Index: A Novel Algorithm for Predicting Antigenic Determinants, CABIOS 4, p. 181-186, 1988; it has been possible so far to detect a preferred sequence having immunogenic activity in the region of the amino acids 7-14 at the N-terminus. Immunization with N-terminal fragments according to established methods predominantly results in antibodies which, as has been described for hPTH(1-34) (J. Tampe, P. Brozio, H.E. Manneck, A. Mißbichler, E. Blind, K.B. Millers, H. Schmidt-Gayk, and F.P. Armbruster, Characterisation of Antibodies Against Human N-Terminal Parathyroid Hormone by Epitope Mapping; J. Immunoassay 13, p. 1-13, 1992), bind in the region of these amino acids. However, these antibodies are not capable of discriminating between biologically active and biologically inactive PTH(1-84) or fragments thereof lacking the first two amino acids serine and valine.

The technical problem which this invention is based upon is to provide peptides by means of which it is possible to eliminate the above-mentioned drawbacks in the diagnosis of biologically active hPTH.

Surprisingly, the technical problem described above is solved by means of the following peptides from the sequence of hPTH(1-37):

hPTH 1-10 (SEQ. ID. NO. 1)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-Gln^6-Leu^7-Met^8-His^9-Asn^{10}-OH (1)

hPTH 1-9 (SEQ. ID. NO. 2)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-Gln^6-Leu^7-Met^8-His^9-OH (2)

hPTH 1-8 (SEQ. ID. NO. 3)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-Gln^6-Leu^7-Met^8-OH (3)

hPTH 1-7 (SEQ. ID. NO. 4)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-Gln^6-Leu^7-OH (4)

hPTH 1-6 (SEQ. ID. NO. 5)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-Gln^6-OH (5)

hPTH 1-5 (SEQ. ID. NO. 6)
NH_2-Ser^1-Val^2-Ser^3-Glu^4-Ile^5-OH (6)

hPTH 9-18 (SEQ. ID. NO. 7)
NH_2-His^9-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (7)

hPTH 10-18 (SEQ. ID. NO. 8)
NH_2-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (8)

hPTH 11-18 (SEQ ID. NO. 9)
NH_2-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (9)

hPTH 12-18 (SEQ. ID. NO. 10)
NH_2-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (10)

hPTH 13-18 (SEQ. ID. NO. 11)
NH_2-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (11)

hPTH 14-18 (SEQ. ID. NO. 12)
NH_2-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-Met^{18}-OH (12)

D hPTH 9-17 SEQ. I.D. NO 13

NH_2-His^{9}-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-Ser^{17}-OH (13)

D hPTH 9-16 SEQ I.D. NO 14

NH_2-His^{9}-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-Asn^{16}-OH (14)

D hPTH 9-15 SEQ. I.D. NO. 15

NH_2-His^{9}-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-Leu^{15}-OH (15)

D hPTH 9-14 SEQ. I.D. NO. 16

NH_2-His^{9}-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-His^{14}-OH (16)

D hPTH 9-13 SEQ. I.D. NO. 17

NH_2-His^{9}-Asn^{10}-Leu^{11}-Gly^{12}-Lys^{13}-OH (17)

D hPTH 24-37 SEQ. I.D. NO. 18

NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (18)

D hPTH 25-37 SEQ. I.D. NO. 19

NH_2-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (19)

D hPTH 26-37 SEQ. I.D. NO. 20

NH_2-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (20)

D hPTH 27-37 SEQ. I.D. NO. 21

NH_2-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (21)

D hPTH 28-37 SEQ. I.D. NO: 22

NH_2-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (22)

D hPTH 29-37 SEQ. I.D. NO. 23

NH_2-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (23)

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D hPTH 30-37, SEQ I.D. NO.24
NH_2-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (24)

D hPTH 31-37, SEQ. I.D. NO.25
NH_2-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (25)

D hPTH 32-37, SEQ. I.D. NO.26
NH_2-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (26)

D hPTH 33-37, SEQ. I.D. NO.27
NH_2-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-Leu^{37}-OH (27)

D hPTH 24-36, SEQ. I.D. NO.28
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-Ala^{36}-OH (28)

D hPTH 24-35, SEQ. I.D. NO.29
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-Val^{35}-OH (29)

D hPTH 24-34, SEQ I.D. NO.30
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-Phe^{34}-OH (30)

D hPTH 24-33, SEQ. I.D. NO.31
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-Asn^{33}-OH (31)

D hPTH 24-32, SEQ. I.D. NO.32
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-His^{32}-OH (32)

D hPTH 24-31, SEQ. I.D. NO.33
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-Asp^{30}-Val^{31}-OH (33)

D hPTH 24-29, SEQ. I.D NO.34
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-Gln^{29}-OH (34)

D hPTH 24-28, SEQ. I.D. NO.35
NH_2-Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}-Leu^{28}-OH (35)

The indicated sequences represent essential features of the secondary structure in their primary structure, as can be demonstrated by supporting NMR data. One precondition to this end was a determination of the PTH(1-37) secondary structure in physiological solution.

The above-mentioned regions of conspicuous structure have good immunogenic activity. Antibodies are formed, binding to the first amino acids of the N-terminus. Deficiency of only two amino acids gives rise to a substantial loss in affinity. Because these amino acids are indispensable for the biological activity to arise, it is possible by using the peptides of the invention to obtain antibodies recognizing only hPTH and fragments thereof which are biologically active.

Furthermore, antibodies can be produced which detect the mid-region 9-15, as well as antibodies giving C-terminal binding in the region of the amino acids 30-37. According to the invention, it is therefore possible to produce antibodies against hPTH(1-37) regions which, according to theoretical calculations, do not exhibit immunogenic activity within the entire molecule. In addition, these regions are separated from each other by such a far distance that no steric hindrance is present which would prevent simultaneous binding of two antibodies.

In preferred embodiments, the peptides may be modified at the N-terminal end, in the side-chain and/or at the C-terminal end, namely, taking the form of acetylation, amidation, phosphorylation and/or glycosylation products.

Eventually, the peptides of the invention may also be bound to carrier proteins such as hemocyanin, thyroglobulin, bovine serum albumin, ovalbumin, or mouse serum albumin etc.. Binding to the carrier proteins is preferably effected using carbodiimide or formaldehyde.

The peptides of the invention may be used in the preparation of a diagnostic agent. The diagnostic agent of the invention can be obtained using the per se known immunization of animals with at least one of the peptides according to the invention. Following immunization, an immunoglobulin fraction can be isolated from the immunized animals, which contains antibody fractions having an antibody titer against at least one of the peptides of the invention. The invention is also directed to the antibodies thus obtained. In addition to the complete antibodies consisting of F_{ab} and F_c, fragments thereof such as F_{ab} or fragments of the antibodies being idiotypes of peptide epitopes may also be used in an alternative embodiment.

The peptides according to the invention are suitable for preparing an agent for the diagnosis of biologically active hPTH(1-37).

Referring to the following examples, the invention will be described in more detail.

Example 1

Solid-Phase Synthesis of Peptides

The method of the invention for synthesizing the peptides is based on the peptide synthesis using a solid support. Each of the C-terminal amino acids is bound to the support material in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine. Wang resin or similar resins are used as support material for the syntheses.

The following derivatives of L-amino acids are used in the synthesis of the sequence, starting from the peptidyl resin as specified: a) hPTH(1-10) (Seq. I.D. No. 1): Fmoc-Asn(Trt)-Wang resin, Fmoc-His(Trt)-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Boc-Ser(tBu)-OH; b) hPTH(9-18) (Seq. I.D. No. 7): Fmoc-Met-Wang resin, Fmoc-Ser(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Boc-His(Trt)-OH; c) hPTH(24-37) (Seq. I.D. No. 18): Fmoc-Leu-Wang resin, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Leu-OH.

The synthesis may be carried out by <u>in situ</u> activation using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) or derivatives thereof, or benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) or derivatives thereof in the presence of diisopropylethylamine or N-methylmorpholine and 1-hydroxybenzotriazole, using a four- to tenfold excess of Fmoc-L-amino acid during the coupling reactions in N,N-dimethylformamide, N,N-dimethylacetamide or N-methylpyrrolidone. Removal of the Fmoc groups is effected using 20% piperidine

or 2% piperidine and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in N,N-dimethylformamide, N,N-dimethylacetamide or N-methylpyrrolidone. Following synthesis, the resins are washed with 2-propanol and dichloromethane and dried to constant weight in a high vacuum.

Removal from the support and deprotection are carried out by reacting the peptidyl resin with trifluoroacetic acid containing 5% scavenger, water, ethanediol, phenol or thioanisole for 30-90 minutes at room temperature, filtrating, washing with trifluoroacetic acid, and subsequently precipitating with tert-butyl methyl ether. The precipitate is lyophilized from aqueous solution.

Example 2

Purification and Analysis

The raw products are purified by chromatography on a C18 reversed phase column (10 µm, buffer A: 0.01 N HCl in water; buffer B: 20% isopropanol, 30% methanol, 50% water, 0.01 N HCl; gradient: 10-80% within 60 minutes; detection at 230 nm).

The purity of the products is determined using mass spectrometry and C18 reversed phase chromatography.

Example 3

Coupling to Carrier Protein

Used as carrier proteins are hemocyanin, thyroglobulin, bovine serum albumin, ovalbumin, or mouse serum albumin. Coupling is performed according to the carbodiimide method by way of the carboxyl groups of the peptides. The peptide is

activated in aqueous solution by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride for 5 minutes. Coupling is effected by adding the activated peptide to an aqueous solution of the carrier. The molar ratio is 1 peptide on 50 amino acids of the carrier protein. The reaction takes 4 hours.